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Note

Maintenance of Mitochondrial DNA by the *Caenorhabditis elegans* ATR Checkpoint Protein ATL-1

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ABSTRACT

Here we show that inactivation of the ATR-related kinase ATL-1 results in a significant reduction in mitochondrial DNA (mtDNA) copy numbers in *Caenorhabditis elegans*. Although ribonucleotide reductase (RNR) expression and the ATP/dATP ratio remained unaltered in *atl-1* deletion mutants, inhibition of RNR by RNAi or hydroxyurea treatment caused further reductions in mtDNA copy number. These results suggest that ATL-1 functions to maintain mtDNA independently of RNR.

PROTEIN kinases in the ataxia-telangiectasia mutated (ATM) family initiate a well-characterized response to DNA damage, resulting in cell-cycle arrest, DNA repair, or apoptosis (Abraham 2001; Shiloh 2003). This family is highly conserved among eukaryotes, comprising two related proteins: ATM and ATR (AT mutant and rad3^{+} related) in humans; Tel1 and Mec1/Esr1 in *Saccharomyces cerevisiae*; Tel1 and Rad3 in *Schizosaccharomyces pombe*; and ATM-1 and ATL-1 in *Caenorhabditis elegans* (Jimenez *et al.* 1992; Kato and Ogawa 1994; Weinert *et al.* 1994; Keith and Schreiber 1995; Lavin *et al.* 1995; Morrow *et al.* 1995; Savitsky *et al.* 1995; Keegan *et al.* 1996; Aoki *et al.* 2000; Boulton *et al.* 2002). These proteins exhibit distinct, but partially overlapping biological functions (Morrow *et al.* 1995; Cliby *et al.* 1998; Ritchie *et al.* 1999). Ataxia telangiectasia (AT) is a human autosomal recessive disease caused by mutations in *ATM*, which result in a wide variety of symptoms; its hallmarks include progressive neuronal degeneration, oculocutaneous telangiectasias, immune dysfunction, predisposition to cancer, incomplete sexual maturation, endocrine abnormalities, and premature aging of the skin and hair (Boder 1975; Shiloh 2001). An aberrant DNA damage response appears to cause immune dysfunction, cancer predisposition, and incomplete sexual maturation, but may not be sufficient to explain all the symptoms of the disease (Shiloh 2001).

In budding yeast, the ATR pathway, governed by Mec1 and Rad53, is essential for cell growth and the DNA damage checkpoint response (Zheng *et al.* 1993; Kato and Ogawa 1994; Weinert *et al.* 1994; Sun *et al.* 1996; Zhao *et al.* 1998). The lethality of *mec1* or *rad53* deletion mutants is suppressed by a mutation in *sml1*, which encodes an inhibitor of ribonucleotide reductase (RNR) (Zhao *et al.* 1998). RNR is a rate-limiting enzyme in *de novo* synthesis of deoxynucleoside triphosphates (dNTP), suggesting that mutation of *sml1* allows *mec1* cells to survive by resulting in increased RNR activity and dNTP levels. Sml1 overproduction frequently causes the formation of petit colonies, due to loss of mitochondria, indicating that decreases in dNTP levels preferentially affect mitochondrial DNA (mtDNA) replication in comparison to chromosomal DNA (chrDNA) replication, which is more mildly affected (Zhao *et al.* 1998). The Mec1 and Rad53 checkpoint pathways, therefore, regulate mtDNA copy number (Taylor *et al.* 2005). However, Sml1-like proteins have not been isolated from other organisms and the effect of ATR on dNTP pools or mtDNA copy number has not been examined in metazoans.

To investigate whether *C. elegans* checkpoint control-related genes participate in mtDNA maintenance, we compared mtDNA copy numbers in wild-type, *atl-1(tm853*, ATR homolog*)*, *atm-1(gk186*, ATM homolog*)*, and *cep-1(w40*, p53 homolog*)* adult hermaphrodites [3 days old from the laid egg (3d): young adult stage] using real-time PCR and normalizing against chrDNA copy number (Sugimoto *et al.* 2008). *atl-1(tm853)* and *atm-1(gk186)* delete a 720-bp region within the exon-7 and a 548-bp region within the parts of intron 1 and exon 2, respectively (http://www.wormbase.org). Both mutations result in frame shifts, which prematurely terminate translation prior to the catalytic center of

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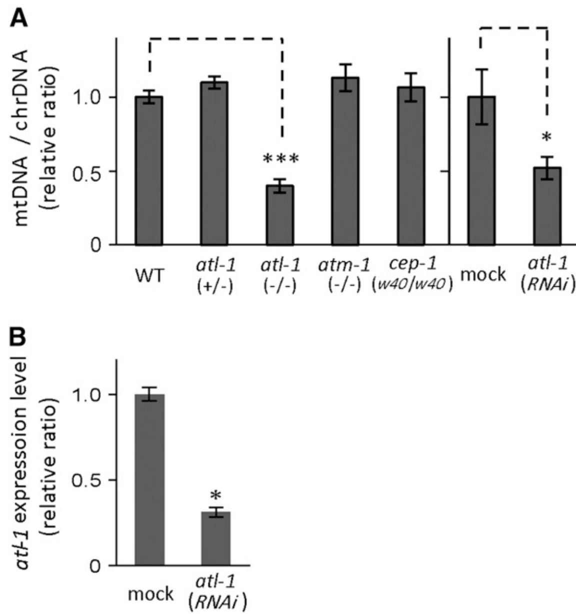

Figure 1.—Relative ratios of mtDNA to chrDNA in adult hermaphrodites with defects in checkpoint-related genes. (A) The mtDNA copy numbers were quantified by normalizing amplifications of mtDNA and chrDNA. *C. elegans* N2 wild-type hermaphrodites, as well as the following strains: JR1279 *cep-1(w40)*; VC381 *atm-1(gk186)*; and HS1208 *atl-1(tm853)*/nT1 (HS1208), prepared from an *atl-1(tm853)*/+ (FX853) and *unc-76*/nT1 male were used. Worm lysates from young adult hermaphrodites (3d) were prepared as described previously (Tsang and Lemire 2002). Real-time quantitative PCR was performed using SYBER Premix Ex Taq (TaKaRa). The primer sets used to amplify mtDNA and chrDNA were described previously (Sugimoto *et al.* 2008). A slight modification was made to the RNAi feeding method (Kamath *et al.* 2001). The *atl-1* RNAi feeding plate was changed twice a day during the parental generation, after which the F_1 progeny were fed on RNAi-containing bacteria during the L4 larval stage, in order to maintain continuous feeding across generations (Sugimoto *et al.* 2008). *Escherichia coli* HT115 (DE3) (Kamath *et al.* 2001) expressing ~930 bp *atl-1* dsRNA (68-1001) cloned with LITMUS28 plasmid vector (New England Biolabs) and *E. coli* HT115 (DE3) harboring the LITMUS28 plasmid (mock control) were used. Experiments were performed on F_2 generations at the young adult stage (24 hr from the L4 larval stage). (B) The expression levels of the *atl-1* gene were monitored by real-time quantitative RT–PCR as compared with the expression level of an elongation factor *eft-2*. Total RNAs were isolated from the young adult stage in the *atl-1* RNAi and mock RNAi. A mock feeding control was performed using *E. coli* HT115 (DE3) harboring the LITMUS28 plasmid. The following primer sets were used to amplify *atl-1*: (forward) 5′-CAG TTT GGC TTC GAT TGC TC and (reverse) 5′-TGA AGC TGG TCC TCT GTC TG, and *eft-2*: (forward) 5′-GAC GCT ATC CAC AGA GGA GG and (reverse) 5′-TTC CTG TGA CCT GAG ACT CC. Real-time PCR experiments were performed in triplicate for each sample. Vertical bars indicate standard error. * and *** indicate statistical significance at $P < 0.05$ and < 0.001, respectively (Student's *t*-test).

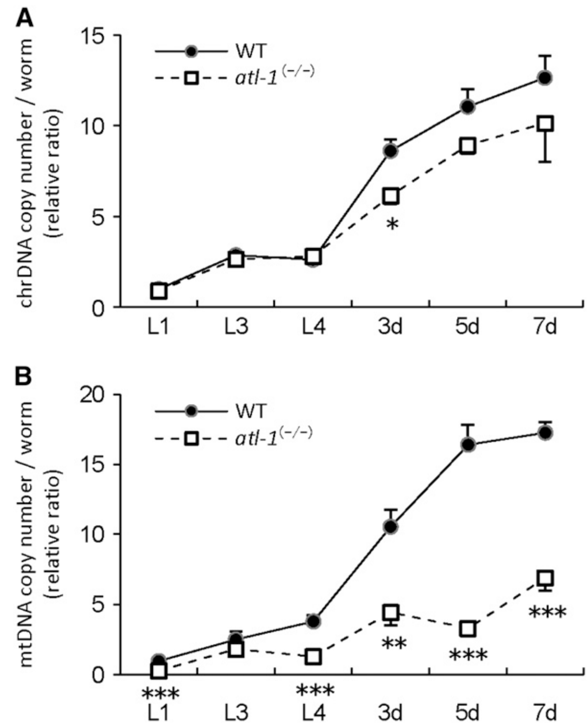

Figure 2.—*atl-1* is required for effective mtDNA replication during germline proliferation. The copy numbers of chrDNA (A) or mtDNA (B) in wild-type hermaphrodites and *atl-1(tm853)* homozygous mutants were measured using real-time PCR; data were normalized relative to wild-type L1 stage larvae. Total DNA was isolated from L1, L3, and L4 larvae, as well as from 3-, 5-, and 7-day-old hermaphrodites. Values represent means obtained from five synchronized but independent worms. Vertical bars indicate standard error. *, **, and *** show statistical significance at $P < 0.05$, <0.01, and < 0.001, respectively (Student's *t*-test).

the respective protein kinase. Thus, both mutations are likely to constitute null alleles (Garcia-Muse and Boulton 2005). The *cep-1(w40)* mutant strain contains an intact copy of *cep-1* at the normal locus, and a 1832-bp deletion encoding a truncated protein lacking the DNA binding domain translocated to elsewhere in the genome (Derry *et al.* 2001). *atl-1(tm853)*, *cep-1(w40)*, and *atm-1(gk186)* homozygotes exhibit defects in DNA damage-induced germ cell apoptosis (Derry *et al.* 2001; Stergiou *et al.* 2007). For *atl-1(tm853)* and *cep-1(w40)*, these defects are dosage sensitive: both *atl-1(tm853)*/+ and *cep-1(w40)*/+ heterozygotes exhibit reduced levels of germ cell apoptosis in response to DNA damage (Derry *et al.* 2001; Stergiou *et al.* 2007). The *atm-1*- and the *cep-1*-defective homozygotes were viable and fertile. Although *atl-1(tm853)* homozygotes appeared to develop into normal adults, their eggs did not hatch, dying during early embryogenesis (Garcia-Muse and Boulton 2005). Garcia-Muse and Boulton (2005) also show that *atl-1(tm853)* causes mitotic catastrophe and loses the S-phase checkpoint and the *atm-1* cooperative checkpoint response to DNA double-strand breaks to induce cell-cycle arrest or apoptosis via the *cep-1* pathway. In addition, *atl-1(RNAi)* affects asymmetric division at the two-cell stage of embryonic development; moreover, *atl-1(RNAi)* frequently produces male (XO) progeny due to nondisjunction of the X chromosome at meiosis I (the Him

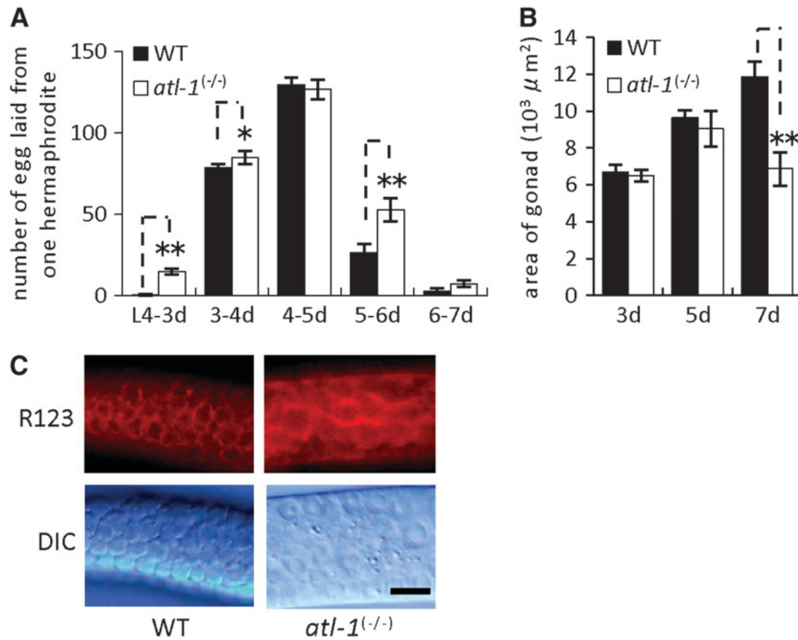

FIGURE 3.—*atl-1* is not required for gonadal development or mitochondrial function. Numbers of eggs laid by a single hermaphrodite (A) and gonadal size from the tip to hinge site (B) in adult hermaphrodites (3, 5, and 7 days old) of wild-type and *atl-1* homozygotes. Vertical bars indicate standard error. * and ** show statistical significance at $P < 0.05$ and < 0.01, respectively (Student's *t*-test). (C) Mitochondrial membrane potential in germ cells from the meiotic pachytene region of wild-type and *atl-1* homozygotes (3-day-old adult hermaphrodites), stained with rhodamine 123 fluorescence, as described previously (SUGIMOTO *et al.* 2008). Bar represents 10 μm.

phenotype, for high incidence of males) (AOKI *et al.* 2000; BOULTON *et al.* 2002; BRAUCHLE *et al.* 2003).

In *atl-1(tm853)* homozygous mutant hermaphrodites, we observed a substantial decrease in relative mtDNA copy number (to less than half of wild-type levels), whereas no reductions were detected in *atl-1(tm853)/+* heterozygotes or mutants defective for *atm-1* and *cep-1* (Figure 1A). Similarly, mtDNA levels decreased significantly (by half) when RNAi feeding was used to silence 80% of *atl-1* expression (Figure 1, A and B).

We examined the copy number of mtDNA and chrDNA in *atl-1(tm853)* and wild-type hermaphrodites at sequential developmental stages (Figure 2). Both mtDNA and chrDNA copy numbers increased from the L4 to the adult stage in wild-type hermaphrodites (Figure 2). In *C. elegans*, somatic cell proliferation is nearly completed prior to hatching (550 somatic cells at hatching *vs.* 959 in adults), and germline proliferation most robustly occurs in the L4 and adult stages (SCHEDL 1997). Maternally derived mtDNA remains unchanged before the early L3 larval stage and thereafter increases significantly in association with germline proliferation (TSANG and LEMIRE 2002). *atl-1(tm853)* homozygotes exhibited a reduced rate of mtDNA accumulation compared to the wild type (Figure 2B). By contrast, chrDNA accumulation was unaffected (Figure 2A). These results suggest that ATL-1 is involved in effective mtDNA replication during germline proliferation.

Following self-fertilization, a single hermaphrodite will lay ~250–300 eggs. After hatching, these individuals grow to adulthood in ~3 days at 20°, passing through four larval stages (L1–L4). The timing and rate of egg production were the same or slightly faster in the *atl-1(tm853)* homozygotes than in the wild-type individuals (Figure 3A), and we observed no significant difference in their gonadal development (Figure 3B). Rhodamine-123 fluorescence staining indicated very similar mitochondrial membrane potentials in the gonads of the *atl-1(tm853)* homozygote and wild-type worms (Figure 3C), despite the former's marked reduction in mtDNA (Figure 2B). *C. elegans* produces more mtDNA than it requires; ~25% mtDNA is sufficient for gonadal development, whereas 10% mtDNA is not (SUGIMOTO *et al.* 2008). Interestingly, *atl-1(tm853)* germ cells were irregular in both shape and size (Figure 3C), presumably as a result of defects in the cell cycle and in chromosome segregation (AOKI *et al.* 2000; GARCIA-MUSE and BOULTON 2005). In the future, it will be important to analyze mitochondrial numbers and morphology in *atl-1(tm853)* mutants.

Depletion or inhibition of RNR activity strongly suppresses mtDNA replication; in contrast, chrDNA replication is less severely affected under normal growth conditions in mammalian cells (EATON *et al.* 2007). RNR contains a large (R1) and small [R2 or p53-inducible R2 (p53R2)] subunit, both of which are essential for maintenance of mtDNA copy number (BOURDON *et al.* 2007; EATON *et al.* 2007). EATON *et al.* (2007) also reported that mammalian ATM regulates RNR expression and human *atm* primary fibroblasts, which were derived from *atm* patients, exhibit lower steady-state levels of R1 and higher levels of p53R2. This decrease in RNR levels in *atm* mutants appears to cause reduced mtDNA copy numbers in actively dividing cells but not in quiescent cells. Similarly, lower R1 levels were found in all tissues of *atm*-deficient knockout mice, but reduced mtDNA levels were only observed in some of these tissues (EATON *et al.* 2007). Therefore, it is difficult to explain fully the control of mtDNA copy number in the mammalian system.

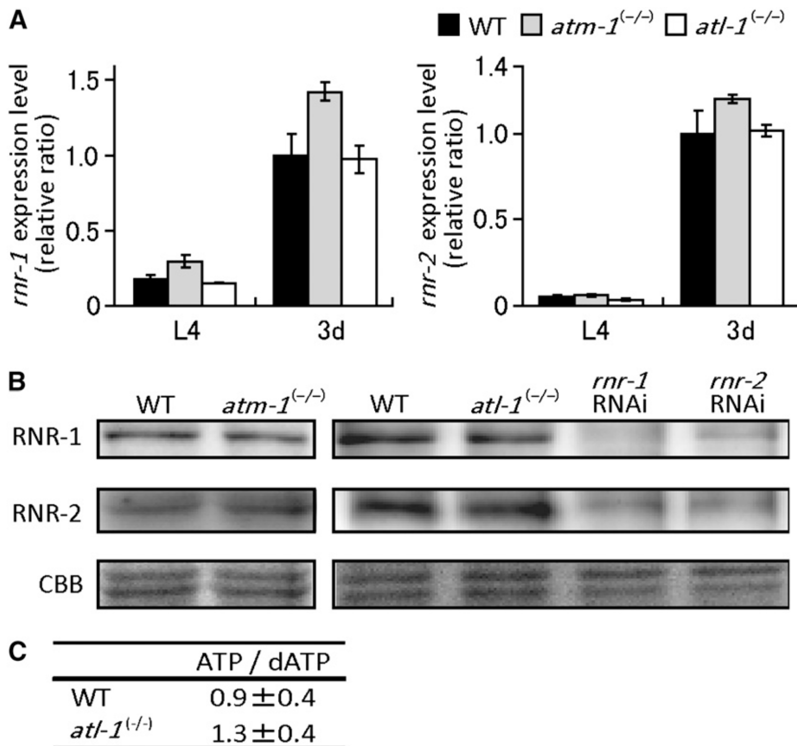

FIGURE 4.—*atm-1* and *atl-1* are not required for RNR expression. (A) The relative transcriptional levels of *rnr-1* and *rnr-2* were normalized using *eft-2* expression. Total RNAs were isolated from L4 and 3-day-old hermaphrodites of wild-type, *atm-1*, and *atl-1* homozygotes. Real-time PCR experiments were performed in triplicate for each sample. The following primer sets were used to amplify *rnr-1*: (forward) 5′-GCG AGT CGA GAA GGA TCA AG and (reverse) 5′-GGC TTC GTA TTT GGC GTA AA, *rnr-2*: (forward) 5′-ATC AGT GCC GAA ACA CTC ATC and (reverse) 5′-CAG CTC ATT CAC CTC CGA CT, and *eft-2* as described in the legend of Figure 1. Vertical bars indicate standard error. (B) Western blot analysis of RNR-1 and RNR-2 protein levels. Wild-type, *atm-1* and *atl-1* homozygous mutant hermaphrodites (3-day-old) and *rnr-1* and *rnr-2* RNAi hermaphrodites were boiled for 5 min in 2× SDS-loading buffer (63 mM Tris-HCl [pH 6.8], 4% SDS, 5% β-mercaptoethanol, 20% glycerol) and then homogenized with a sonicator for 30 sec. The RNAi samples were obtained from the young adults (3 days from L1) that had been fed from the L1 larval stage on *E. coli* HT115 (DE3) expressing *rnr-1* dsRNA (541-1559) or *rnr-2* dsRNA (254-1397) (Kamath *et al.* 2001). Total protein from 25 adult hermaphrodites was separated by discontinuous 8 or 12% SDS–PAGE and then electrotransferred to Hybond ECL nitrocellulose membranes (GE Healthcare). Immunochemical hybridization was performed using anti-RNR-1 (Santa Cruz Biotechnology) and anti-RNR-2 (Calbiochem) polyclonal antibodies, with horseradish peroxidase-coupled secondary antibodies of donkey anti-goat IgG (AP-180P, Chemicon) and goat anti-rabbit IgG (GE Healthcare), respectively. Signals were visualized using the ECL Plus Western blotting detection system (GE Healthcare) and quantified with a Fuji LAS 1000 digital image analyzer (Fuji film). Coomassie Brilliant Blue (CBB) staining of major proteins was used as an internal standard. (C) The ATP/dATP ratios of wild-type hermaphrodites and *atl-1(tm853)* homozygous mutants were measured using LC-MS/MS. Approximately 1000 wild-type hermaphrodites and *atl-1(tm853)* homozygous mutants (3 days old) were suspended in PBS containing 2% SDS and boiled for 5 min, followed by homogenization with a sonicator for 30 sec. The extract was deproteinized with TCA (final concentration of 5%), vortexed, placed in an ice bath to incubate for 10 min, and then revortexed. The supernatant was collected, neutralized with potassium carbonate, and filtered through a 0.45 μm membrane filter. Finally, the extract was purified by centrifugal ultrafiltration using a 30,000 MW cutoff membrane (Amicon YM-3, Millipore).

C. elegans RNR comprises two subunits, RNR-1 (large subunit) and RNR-2 (small subunit) (Hong *et al.* 1998; van den Heuvel 2005). In addition, its genome contains the gene *F19G12.2*, which encodes a protein with greater similarity to mammalian p53R2 than R2. Although *F19G12.2* expression is not induced by the DNA damage response, it is strongly expressed in males and L1 hermaphrodite larvae, but found rarely in adult hermaphrodites (Jiang *et al.* 2001; Roy *et al.* 2002). Therefore, it appears that p53R2 may be a vertebrate-specific protein. To determine whether the *C. elegans* AT family proteins regulate expression of either *rnr-1* or *rnr-2*, we performed quantitative analyses of gene expression using real-time RT–PCR and Western blots. We observed significant upregulation of *rnr-1* and *rnr-2* transcription during germline proliferation in wild-type hermaphrodites between the L4 larval and young adult stages (Figure 4A). Transcriptional upregulation occurred normally in *atm-1(gk186)* and *atl-1(tm853)* mutant homozygotes (Figure 4A) and Western blot analyses did not reveal reductions in RNR-1 or RNR-2 protein levels in *atm-1(gk186)* or *atl-1(tm853)* mutants (Figure 4B). We also measured ATP/dATP ratios in *atl-1(tm853)* mutants using LC-MS/MS on an API-400 instrument (Applied Biosystems). We observed no significant differences between the ATP/dATP ratios of wild-type and *atl-1(tm853)* homozygotes (P-value = 0.54, Student's t-test, Figure 4C). In contrast, in *S. cerevisiae*, dNTP levels increase twofold in *sml1* null mutants (Zhao *et al.* 1998). Taken together, these results suggest that *C. elegans* ATL-1 affects mtDNA copy number without affecting the steady state RNR levels.

To determine if *C. elegans* RNR activity affects mtDNA copy numbers, we performed knockdown of *rnr-1* or *rnr-2* expression by feeding animals with gene-specific RNAi and treatment of an RNR inhibitor, hydroxyurea (HU). Measurement of mRNA levels for *rnr-1* or *rnr-2* showed that the RNAi treatment was efficacious, reducing *rnr-1* mRNA levels by 70% and *rnr-2* mRNA levels by 90% (Figure 5A). Both *rnr-1(RNAi)* and *rnr-2(RNAi)* resulted in reductions in the mtDNA copy number (Figure 5B). Both *rnr-1(RNAi)* and *rnr-2*(*RNAi*) hermaphrodites also exhibited a protruding vulva and sterility (data not shown). At the protein level, RNR-1 and RNR-2 subunits

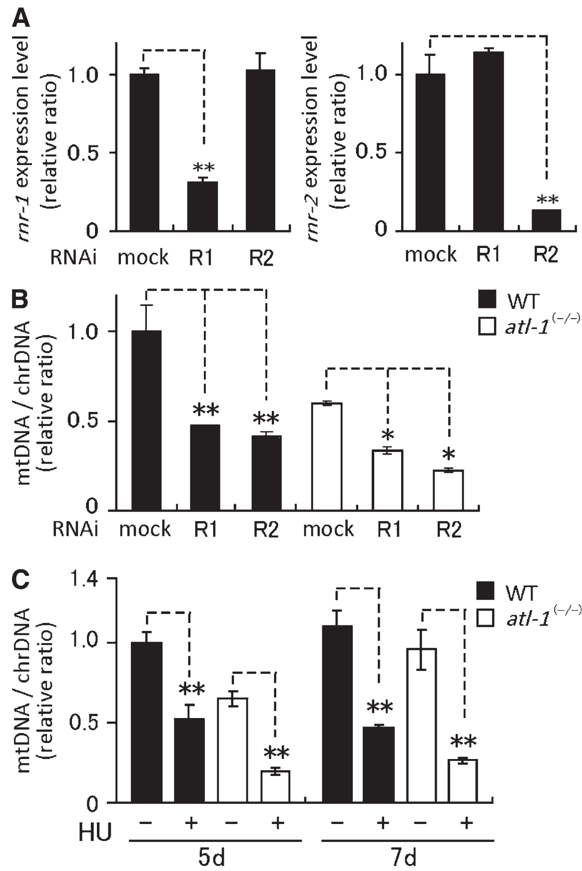

Figure 5.—Depletion or inhibition of RNR causes a reduction in mtDNA copy number. (A) The expression levels of the *rnr-1* and *rnr-2* genes in either *rnr-1(RNAi)* (R1) or *rnr-2(RNAi)* (R2) were monitored by real-time quantitative RT–PCR as compared with the expression level of an elongation factor *eft-2*. The primer sets were used as described in the legend of Figure 4. (B) Relative ratios of mtDNA to chrDNA copy numbers in wild-type and *atl-1(tm853)* homozygotes with RNAi-depleted *rnr-1* or *rnr-2*. (C) Effect of HU treatment on mtDNA copy number in wild-type and *atl-1(tm853)* hermaphrodites. Three-day-old young adult hermaphrodites were cultured for 2–4 days on NGM plates containing 30 mm HU, and then mtDNA and chrDNA were isolated from 5- and 7-day-old hermaphrodites. Real-time PCR amplifications were performed in triplicate for each sample. Vertical bars indicate standard error. * and ** show statistical significance at $P < 0.05$ and < 0.01, respectively (Student's *t*-test).

appear to be coordinately regulated, as *rnr-1(RNAi)* reduced RNR-2 levels and *rnr-2(RNAi)* reduced RNR-1 levels (Figure 4B). Since *rnr-1(RNAi)* does not affect *rnr-2* mRNA levels, and vice versa (Figure 5A), complex formation may be important for the accumulation of both RNR subunits. The treatment of wild-type hermaphrodites with 30 mm HU resulted in a reduction of mtDNA at each developmental stage (Figure 5C). Following RNR inhibition by HU treatment or *rnr-1(RNAi)* or *rnr-2(RNAi)*, *atl-1(tm853)* homozygotes exhibited further reductions in mtDNA levels (Figure 5, B and C). Taken together, our results indicate that *C. elegans* RNR is necessary for mtDNA replication during germline proliferation, but that RNR levels are not dependent on ATL-1 function. Thus, ATL-1 likely affects mtDNA levels by a different mechanism, the nature of which is unclear.

In addition, we used quantitative real-time RT–PCR to investigate whether or not ATL-1 regulates the transcriptional levels of other proteins involved in mtDNA replication, including DNA polymerase gamma (*Y57A10A.15* gene), mitochondrial single-stranded DNA-binding protein (*mtss-1*), and the predicted mitochondrial transcription factor A (*hmg-5*). However, we found no evidence to indicate that ATL-1 regulates their transcription (*P*-values were 0.94, 0.68, and 0.88, respectively).

Mammalian ATM regulates mtDNA copy number through RNR expression levels, and p53R2 is essential for maintenance of mtDNA (Bourdon *et al.* 2007; Eaton *et al.* 2007). In addition, Fu *et al.* (2008) recently reported that phosphorylation of AMP-activated protein kinase by ATM controls mitochondrial biogenesis in response to DNA damage. In multicellular organisms, there is growing evidence of mtDNA maintenance controlled by checkpoint related proteins but not ATR. Our study suggests that the *C. elegans* checkpoint protein ATL-1 participates in mtDNA replication by a mechanism that is separate from the control of dNTP pools or RNR protein levels.

ATR-type proteins are checkpoint factors that control the nuclear DNA replication fork by phosphorylation of proteins such as RPA2, which binds to nuclear single-stranded DNA (Olson *et al.* 2006). This work provides some insight into the roles played by ATR in maintaining mtDNA copy numbers in higher eukaryotes. It is possible that ATL-1 regulates the efficiency of mtDNA replication by phosphorylating protein(s) involved in the replication process, such as DNA polymerase γ and mitochondrial single-stranded DNA binding protein. Mitochondria contain their own DNA, which encodes proteins that are essential for the respiratory chain machinery. Thus, mitochondria must undergo DNA replication prior to cell proliferation. Since the copy number of mtDNA increases during late G_0/G_1 and early S phase (Trinei *et al.* 2006), specific signals must stimulate mtDNA replication to enable synchronization with the cell cycle. Our future experiments will focus on the molecular mechanism(s) underlying the coordination of mtDNA and chrDNA replication by ATR.

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